Postharvest heat and conditioning treatments activate different molecular responses and reduce chilling injuries in grapefruit

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Abstract

A combination of hot water (a rinse at 62 °C for 20 s) and conditioning (pre-storage at 16 °C for 7 d) treatments synergistically reduced chilling injury development in grapefruit (Citrus paradisi, cv. ‘Star Ruby’) during cold storage at 2 °C, suggesting that the treatments may activate different chilling tolerance responses. To study the molecular mechanisms involved, chilling- and conditioning-responsive genes were isolated by polymerase chain reaction (PCR) cDNA subtraction, cDNA libraries were constructed from hot water- and conditioning-treated fruit, and cDNA sequencing was used to identify putative stress-responsive and chilling tolerance genes. PCR cDNA subtraction revealed the identification of 17 chilling-responsive and heat- and conditioning-induced genes, and the expression patterns of 11 additional stress-related genes, antioxidant defensive genes, and genes encoding enzymes involved in membrane lipid modifications were characterized. It was found that hot water and conditioning treatments had little effect on gene expression by themselves, but rather had a priming effect, and enabled the fruit to activate their defense responses after subsequent exposure to chilling. RNA gel blot hybridizations revealed that the expression patterns of eight genes, including HSP19-I, HSP19-II, dehydrin, universal stress protein (USP), EIN2, 1,3;4β-D-glucanase, and superoxide dismutase (SOD), were specifically regulated by the heat treatment, and four genes, including fatty acid desaturase2 (FAD2) and lipid transfer protein (LTP), were specifically regulated by the conditioning treatment. Furthermore, four more genes were identified, including a translation initiation factor (SUI1), a chaperonin, and alcohol dehydrogenase (ADH), that were commonly regulated by both heat and conditioning treatments. According to these data, it is suggested that pre-storage heat and conditioning treatments may enhance fruit chilling tolerance by activating different molecular mechanisms. The hot water treatment activates mainly the expression of various stress-related genes, whereas the conditioning treatment activates mainly the expression of lipid membrane modification enzymes.

Key words: Chilling, conditioning, grapefruit, hot water, postharvest, stress.

Introduction

Many plant species, especially those of tropical and subtropical origin, are severely injured or killed by exposure to low non-freezing temperatures (Lyons, 1973; Lynch,
1990). Exposure of these chilling-sensitive plants to low growth temperatures often results in numerous cellular and metabolic dysfunctions, such as altered respiration rates, impaired photosynthetic activity, and changes in membrane permeability (Lyons, 1973; Graham and Patterson, 1982; Allen and Ort, 2001).

In citrus, as with many other horticultural commodities, chilling sensitivity also imposes a major limitation on the postharvest handling of the fruits, since, for some cultivars, such as grapefruit, it necessitates storage at relatively high temperatures of 11–13 °C (Kader and Arpaia, 2002). Exposure of grapefruit to lower temperatures of 0–8 °C results in the development of chilling injuries (CIs), which appear as dark sunken lesions (pitting) of collapsed tissues throughout the peel surface (Porat, 2004) (Fig. 1).

In previous studies, it was found that pre-storage heat and temperature conditioning treatments enhanced chilling tolerance in citrus fruits and reduced the development of CI during postharvest storage (Porat et al., 2000a). The recommended low temperature conditioning treatment to reduce CI in citrus fruit includes exposure to 16 °C for 7 d before transfer to low temperatures (Hatton and Cubbedge, 1982, 1983). In addition, it was previously shown that a short pre-storage hot water rinsing and brushing (HWB) treatment at 62 °C for 20 s also effectively reduced CI development (Porat et al., 2000a, b).

As compared with plant responses to other abiotic stresses, such as freezing, drought, salinity, and heat, only little is known as yet regarding the molecular basis of chilling tolerance, or of the signal transduction networks involved in its acquisition. Nevertheless, it was previously reported that activation of genes encoding enzymes involved in membrane lipid modifications, antioxidant defensive genes, and possibly other known stress-responsive genes may be involved in the enhancement of chilling tolerance. For example, Arabidopsis plants mutated in various fatty acid desaturase (FAD) genes (fad2, fad5, and fad6) became chilling sensitive (Somerville and Browse, 1991; Miquel et al., 1993), whereas overexpression of FAD genes, or of other genes, such as acetyltransferases and phospholipid translocases, which increase membrane fluidity, correspondingly increase chilling tolerance (Murata et al., 1992; Ishizaki-Nishizawa et al., 1996; Goméz et al., 2000). In addition, it was demonstrated that overexpression of antioxidant defensive genes, such as ascorbate peroxidase (APX), superoxide dismutase (SOD), and catalase (CAT), increased chilling tolerance (Van Breusegem et al., 1999; Payton et al., 2001), whereas repression of CAT gene expression reduced it (Kerdnaimongkol and Woodson, 1999). Finally, several reports have suggested that various stress genes, usually related to other types of stress responses, may also be involved in the acquisition of chilling tolerance. For instance, it was suggested that particular heat shock proteins (HSPs) (Sabehat et al., 1998; Sung et al., 2001) and dehydrin genes (Ismail et al., 1999) may also be involved in conferring chilling tolerance on plants.

In previous studies, it was shown that application of the pre-storage HWB treatment at 62 °C for 20 s, which effectively increased chilling tolerance, also induced the expression of various stress-related genes in grapefruit flavedo tissue (the outer coloured layer of the peel). For example, it was found that HWB followed by storage at 2 °C increased the expression of two dehydrin genes (COR15 and cpDHN), four HSPs (HSP18-I, HSP18-II, HSP22, and HSP70), and cNHX1, a sodium proton antiport gene known to be involved in salt tolerance in plants (Porat et al., 2002a, b, 2004; Rozenzvig et al., 2004). Furthermore, other studies showed that a high temperature heat conditioning treatment at 37 °C for 3 d significantly increased chilling tolerance in ‘Fortune’ mandarins and correspondingly induced the expression of the WRKY and TFIIIB transcription factors and of several stress genes, such as chitinases and cell wall hydrolysis enzymes (Sanchez-Ballesta et al., 2003).

In the present study, it was found that postharvest low temperature conditioning and heat treatments activated apparent different chilling tolerance-responsive pathways in grapefruit and synergistically prevented the development of CI. Furthermore, by using two different molecular approaches—construction of polymerase chain reaction (PCR) cDNA subtraction libraries and conduction of a grapefruit flavedo expressed sequence tag (EST) sequencing project—new chilling-responsive and HWB- and conditioning-induced genes were identified that are linked

Fig. 1. CI damage (pitting) in grapefruit. The chilling damage is manifested as dark sunken areas of collapsed tissue (pitting) all over the peel surface.
with the acquisition of chilling tolerance in citrus. In all, it was shown that postharvest heat and conditioning treatments activate different molecular mechanisms, which suggests that several different defensive pathways against low temperature stress may be involved in the acquisition of chilling tolerance in fruits.

Materials and methods

Plant material and CI evaluation
Grapefruits (Citrus paradisi, cv. ‘Star Ruby’) were obtained from commercial orchards and used on the day after harvest. Afterwards, the fruits were stored at 2 °C for 8 or 14 weeks under a relative humidity of ~90%. Each treatment included four boxes, each containing 30 fruit (total of 120 fruit per treatment). The experiments were performed twice, during two consecutive seasons. For CI evaluations, fruit were sorted into four categories according to their CI severity: none (score 0; no pitting), slight (score 1; a few scattered pits), moderate (score 2; pitting covering up to 30% of the fruit surface), and severe (score 3; extensive pitting covering >30% of the fruit surface). The CI index was determined for each treatment by multiplying the number of fruits in each category by their score, and then dividing this sum by the total number of fruits assessed.

Postharvest HWB and conditioning treatments
Hot water treatments at 62 °C for 20 s were applied by rinsing the fruit as they moved along a set of brush rollers, as described previously (Porat et al., 2000a, b). The temperature conditioning treatment was applied by keeping the fruit at 16 °C in a separate storage room for 7 d, and then transferring them to continuous storage at 2 °C (Hatton and Cubbedge, 1982, 1983; Porat et al., 2000a).

Isolation of RNA
Total RNA was isolated from grapefruit flavedo by phenol/chloroform extraction and precipitation with LiCl according to standard procedures (Sambrook et al., 1992). Poly(A)⁺ RNA was isolated from total RNA by using the PolyATtract mRNA Isolation Systems Kit (Promega, Madison, WI, USA).

Construction of cDNA libraries and sequence analysis
HWB- and conditioning-induced grapefruit flavedo cDNA libraries were constructed from 5 μg of poly(A)⁺ RNA isolated 24 h after HWB or immediately following the conditioning treatment (7 d at 16 °C). The cDNA libraries were constructed with the ZAP-cDNA synthesis kit (Strategene, La Jolla, CA, USA) according to the manufacturer’s instructions.

Sequencing was performed by the dideoxynucleotide chain termination method (Sanger et al., 1977), and sequence analysis was done with the BLAST computer programs (Altschul et al., 1997).

PCR cDNA subtraction analysis
PCR cDNA subtraction was performed with the CLONTECH PCR-Select cDNA Subtraction Kit (Clontech, Palo Alto, CA, USA) according to the manufacturer’s instructions. In general, the subtraction analysis was performed twice with cDNA synthesized from 2 μg of poly(A)⁺ RNA isolated from either control (driver) or treated (tester) fruits immediately after the conditioning treatment (7 d at 16 °C) and after two more weeks in cold storage (7 d at 16 °C+2 weeks at 2 °C). The final subtracted cDNA fragments were cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA).

Results

Heat and conditioning treatments synergistically reduce CI
CI is a major postharvest physiological disorder in citrus fruit, and causes considerable economic losses. It is manifested as dark sunken areas of collapsed tissue (pitting) all over the peel surface (Fig. 1). In the present study, it was found that HWB and conditioning treatments applied separately effectively reduced the development and severity of CI by 85% and 83%, respectively, after 8 weeks of storage at 2 °C. After a much longer period of 14 weeks at 2 °C, the CI index was reduced by 64% and 56%, respectively, (Fig. 2). However, it was found that the combined application of the two treatments was much more effective than the application of either treatment alone, and significantly reduced the development of CI by 98% and 93% after 8 weeks and 14 weeks, respectively (Fig. 2).

Fig. 2. Effects of HWB and conditioning treatments on the development of CI in grapefruit. The CI percentages were measured after 8 weeks (A) and 14 weeks (B) of storage at 2 °C. Data are means of four replications per treatment, each containing 30 fruits (total of 120 fruit per treatment).
In order to identify genes that might be involved in the acquisition of chilling tolerance of grapefruit, two PCR cDNA subtraction libraries were constructed with RNA isolated from the peel of control fruits at time zero (driver cDNA) and from the peel of conditioned fruits at two different time points: immediately after conditioning (7 d at 16 °C) or after two additional weeks in cold storage (7 d at 16 °C+2 weeks at 2 °C) (tester cDNAs). Sequencing, nucleotide BLAST searches, and confirmation by RNA gel blot hybridization analysis of ~70 randomly chosen PCR cDNA fragments from each subtracted cDNA library revealed the identities of 17 cDNAs that were specifically induced either by chilling itself (exposure to 2 °C) or following application of the hot water or conditioning treatments (Table 1).

Only one clone (S8, similar to an unknown protein from tomato) was isolated from the first subtracted library, which was constructed immediately after the conditioning treatment, whereas all the other cDNAs were isolated from the second cDNA subtracted library, which was constructed after two additional weeks of exposure to 2 °C. Among these identified cDNAs, seven clones (S18, S26, S28, S41, S49, S78, and S80) were homologous to other genes with known functions, whereas the other 10 were either novel or similar to other genes with unknown functions (Table 1).

### Isolation of grapefruit stress-related, antioxidant defense and lipid modification genes by EST sequencing

In an effort to identify, in grapefruit, stress-responsive genes, antioxidant defensive genes, and genes encoding enzymes involved in membrane lipid modifications that may be influenced by the HWB and conditioning treatments, two grapefruit flavedo cDNA libraries were constructed, the first 24 h after application of the HWB treatment, and the second immediately after the conditioning treatment (7 d at 16 °C), and an EST sequencing project was initiated at the US Horticulture Research Laboratory Genomic Center at Ft Pierce, FL, USA. After ~1550 randomly chosen, different cDNAs from the HWB-treated cDNA library and ~550 cDNAs from the conditioned cDNA library had been sequenced, the expression patterns of 11 different cDNAs were evaluated following exposure of the fruits to the heat and conditioning treatments and subsequent cold storage (Table 2). These chosen cDNAs comprised six stress-responsive genes—alcohol dehydrogenase (ADH) (anaerobic stress), dehydrin, and a dehydration-induced protein (DIP) (drought stress), HSP19-I and HSP19-II (heat stress), and universal stress protein (USP) (general stress responsive)—three antioxidant defensive genes—APX, CAT, and SOD—and two genes encoding the lipid modification enzymes—FAD2 and lipid transfer protein (LTP) (Table 2).

### Characterization of chilling-, heat-, and conditioning-responsive genes by RNA gel blot hybridizations

In order to characterize the possible involvement of the various isolated genes in heat- and conditioning-induced chilling tolerance responses in grapefruit, their expression patterns were evaluated in response to the application of each treatment alone, without subsequent cold storage. It can be seen that among the 28 cDNAs tested, expression patterns of only 13 cDNAs were affected by the applications of the HWB or the conditioning treatment by themselves (Fig. 3). Among these, the expression patterns of three genes were induced by either treatment (S34, Al-induced protein, and S70), the expression of five was specifically induced by HWB (HSP19-I, HSP19-II, TRX, S50, and S84), and two were specifically induced by the conditioning treatment (S8 and FAD2) (Fig. 3). Furthermore, it was found that the

### Table 1. List of chilling-inducible, HWB-responsive, and conditioning-responsive grapefruit flavedo cDNAs identified by PCR cDNA subtraction analysis

<table>
<thead>
<tr>
<th>Clone name</th>
<th>GenBank accession number</th>
<th>Homology to known genes</th>
<th>E-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>S4</td>
<td>DQ056320</td>
<td>Hypothetical protein, <em>A. thaliana</em> (BAB10956.1)</td>
<td>9e-17</td>
</tr>
<tr>
<td>S8</td>
<td>DQ056321</td>
<td>Clone 132586R, <em>Lycopersicon esculentum</em> (BTO13727)</td>
<td>4e-04</td>
</tr>
<tr>
<td>S18</td>
<td>AY271308</td>
<td>Thioredoxin h. <em>C. paradisi</em></td>
<td>0.0</td>
</tr>
<tr>
<td>S26</td>
<td>DQ056322</td>
<td>Lapase class 3-like, <em>Oryza sativa</em> (BAD35707.1)</td>
<td>8e-27</td>
</tr>
<tr>
<td>S28</td>
<td>DQ056323</td>
<td>SU11 protein, <em>Coffeea arabica</em> (CAD58629.1)</td>
<td>6e-33</td>
</tr>
<tr>
<td>S34</td>
<td>DQ056324</td>
<td>Unknown protein</td>
<td></td>
</tr>
<tr>
<td>S41</td>
<td>DQ056325</td>
<td>Chaperonin, putative, <em>A. thaliana</em> (NP200461.2)</td>
<td>4e-38</td>
</tr>
<tr>
<td>S43</td>
<td>DQ056326</td>
<td>Unknown protein</td>
<td></td>
</tr>
<tr>
<td>S44</td>
<td>DQ056327</td>
<td>Unknown protein</td>
<td></td>
</tr>
<tr>
<td>S49</td>
<td>DQ056328</td>
<td>Al-induced protein, <em>Gossypium hirsutum</em> (AAQ74889.1)</td>
<td>4e-45</td>
</tr>
<tr>
<td>S50</td>
<td>DQ056329</td>
<td>Unknown protein</td>
<td></td>
</tr>
<tr>
<td>S59</td>
<td>DQ056330</td>
<td>Unknown protein</td>
<td></td>
</tr>
<tr>
<td>S62</td>
<td>DQ056331</td>
<td>Unknown protein</td>
<td></td>
</tr>
<tr>
<td>S70</td>
<td>DQ056333</td>
<td>Unknown protein</td>
<td></td>
</tr>
<tr>
<td>S78</td>
<td>DQ056334</td>
<td>EIN2, <em>Petunia hybrida</em> (AAR08678.1)</td>
<td>2e-29</td>
</tr>
<tr>
<td>S80</td>
<td>DQ056335</td>
<td>Putative endo-1,3,4-β-D-glucanase, <em>Oryza sativa</em> (AAU10802.1)</td>
<td>1e-55</td>
</tr>
<tr>
<td>S84</td>
<td>DQ056336</td>
<td>Unknown protein</td>
<td></td>
</tr>
</tbody>
</table>
expression patterns of two genes were specifically down-regulated by HWB (S8 and DIP), and those of another two were specifically down-regulated following the conditioning treatment (EIN2 and dehydrin) (Fig. 3).

Following exposure of the fruit to low temperature (storage at 2°C), it was found that the expression patterns of nine genes were affected by exposure to chilling itself, no matter whether they had received the pre-storage hot water or conditioning treatments that enhance chilling tolerance in fruits (Fig. 4). Among these, the expression patterns of seven genes were up-regulated by chilling (TRX, lipase, CAT, S4, S43, S50, and S62), and two were down-regulated by chilling (S44 and DIP) (Fig. 4). These nine chilling-responsive genes are probably part of the fruit’s basal low-temperature defensive system, but not part of the HWB- or conditioning-induced chilling tolerance pathways.

In contrast to these general chilling-responsive genes, several genes were identified that were specifically regulated by cold storage that followed the application of the HWB or the conditioning treatment, but not by cold storage alone (Fig. 5). Among these, eight genes were identified that were specifically regulated by HWB followed by cold storage (Fig. 5A), four by conditioning followed by cold storage (Fig. 5B), four regulated by either HWB or by conditioning, followed by cold storage (Fig. 5C), and two specifically regulated by the combination of HWB and conditioning, followed by cold storage (Fig. 5D). The HWB-specific genes included up-regulation of five genes (dehydrin, HSP19-I, HSP19-II, USP, and S84) and down-regulation of three (EIN2, 1,3,4-β-d-glucanase, and SOD) (Fig. 5A); the conditioning-specific genes included up-regulation of four genes (S8, S59, FAD2, and LTP) (Fig. 5B); the common HWB- and conditioning-regulated genes included up-regulation of two genes (Al-induced protein and S70) (Fig. 5D).

Interestingly, it was found that the induction of most genes that were specifically induced by either the HWB or conditioning treatment during cold storage required
further exposure to chilling, but these genes were not induced by the application of either treatment by itself without subsequent cold storage (Table 3). For example, among the eight different HWB-regulated genes, only \textit{HSP19-I} and \textit{HSP19-II} were induced by exposure to hot water alone, and, among the four conditioning-regulated genes, only \textit{S8} and \textit{FAD2} were affected by exposure to the conditioning treatment alone (Figs 3, 5; Table 3). Among the HWB and conditioning commonly regulated genes found, only clone \textit{S34} was induced by exposure to either treatment alone without subsequent cold storage (Figs 3, 5; Table 3). Thus, the HWB and conditioning treatments mainly had a ‘priming’ effect, and enabled the fruits to activate most of their defence responses after the subsequent exposure to chilling.

Overall, these RNA hybridization studies revealed that pre-storage hot water and conditioning treatments specifically activated different sets of genes in grapefruit peel tissue during cold storage (Fig. 6). Moreover, it can be seen that the HWB treatment mainly effected the expression of various stress-related genes (such as \textit{HSP19-I}, \textit{HSP19-II}, dehydrin, \textit{USP}, \textit{SOD}, \textit{EIN2}, and glucanase), whereas the conditioning treatment mainly activated the expression of genes encoding enzymes involved in membrane lipid modifications (such as \textit{FAD2} and \textit{LTP}) (Fig. 6). In addition to heat-specific and conditioning-specific gene expression pathways, both treatments also commonly activated another set of genes (including \textit{SUII}, \textit{ADH}, and \textit{chaperonin}) which may have a common role in conferring fruit chilling tolerance (Fig. 6).

**Discussion**

In this study, it was found that the combined application of HWB and conditioning treatments was much more effective in reducing the development of CI in grapefruit than the application of each treatment alone, which suggests that the two treatments activated different chilling-stress defensive pathways (Fig. 1). This finding appears to be a novel observation that a synergistic effect between two different treatments can increase chilling tolerance.

Overall, nine chilling-responsive genes were identified in grapefruit, whose expression patterns were affected by exposure to chilling, independently of the treatments that the fruits had received after harvest (Fig. 4). These chilling-responsive genes included four known genes, \textit{TRX}, lipase, \textit{CAT}, and \textit{DIP}, and five genes of unknown function (Fig. 4). It is suggested that these chilling-responsive genes may be part of a basal low temperature defensive pathway of the fruits, and are probably involved in their natural attempt to cope with exposure to chilling. Among these genes, \textit{TRX} participates in redox regulation of selected target proteins, including oxidative defensive enzymes, such as peroxiredoxins (Schurmann and Jacquot, 2000). Lipases catalyse the hydrolysis of fatty acids from phospholipids,

![Fig. 4](https://academic.oup.com/jxb/article-abstract/57/12/2943/442312) Effects of chilling, HWB, and conditioning treatments on the mRNA levels of putative chilling tolerance genes in grapefruit flavedo. Each lane contained 10 µg of total RNA isolated after 0, 1, 2, 3, 4, 6, or 9 weeks of exposure to 2 °C. Ethidium bromide staining of the RNA gel was used to ensure equal loading among lanes.
Fig. 5. Effects of chilling, HWB, and conditioning treatments on the mRNA levels of putative chilling tolerance genes in grapefruit flavedo. Each lane contained 10 μg of total RNA isolated after 0, 1, 2, 3, 4, 6, or 9 weeks of exposure to 2 °C. The various genes were classified into different groups according to their expression patterns: (A), genes specifically regulated by HWB; (B), genes specifically regulated by conditioning; (C), genes regulated by either HWB or conditioning, (D), genes specifically regulated by the combination of HWB and conditioning. Ethidium bromide staining of the RNA gel was used to ensure equal loading among lanes.
and their activity provides the first limiting step in the biosynthesis pathway of the plant growth regulator jasmonic acid, which is known to be involved in the activation of plant defence responses to exposure to environmental stresses (Schaller, 2001; Howe and Schilmiller, 2002). Furthermore, it was recently suggested that lipases may be involved in oxidative stress defence responses by specifically removing damaged peroxidized fatty acids from the membrane bilayer (Lo et al., 2004). The CAT gene encodes a major antioxidant defence enzyme (Scandalios, 1990), well known to be involved in the acquisition of chilling tolerance in many horticultural crops including citrus (Sala and Lafuente, 2000; Payton et al., 2001).

The HWB-induced chilling tolerance-responsive pathway explored in this study comprises the expression of various stress-related genes, including up-regulation of HSP19-I, HSP19-II, dehydrin, and USP, and down-regulation of SOD, glucanase, and the ethylene signal transduction component EIN2 (Fig. 5A). The HWB treatment had no effect on the transcript levels of APX (data not shown). HSPs function as molecular chaperones and assist in protein folding, assembly and transport, and targeting of damaged proteins for proteolysis; thus, they may also assist in protecting the cells under chilling stress conditions (Vierling, 1991; Wang et al., 2004). Dehydrins act as structural stabilizers with suggested chaperone-like properties and protect various nuclear and cytoplasmic macromolecules from coagulation during dehydration (Close, 1997). In previous studies, it was suggested that dehydrins might be involved in conferring chilling tolerance on cowpea, tobacco, and citrus (Ismail et al., 1999; Porat et al., 2002a; Hara et al., 2003). USPs are conserved proteins among bacteria, fungi, algae, yeast, and plants, and are thought to be involved in protecting cells from oxidative stress damage (Kvint et al., 2003). SOD encodes a major antioxidant defensive enzyme involved in detoxification of superoxide radicals (Scandalios, 1990).

### Table 3. Expression patterns of genes affected by either the HWB or conditioning treatments alone or followed by exposure to chilling (2 °C)

<table>
<thead>
<tr>
<th>Clone</th>
<th>Heat alone</th>
<th>Heat+chilling</th>
<th>Conditioning alone</th>
<th>Conditioning+chilling</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genes regulated by HWB followed by chilling</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSP19-I</td>
<td>√</td>
<td>√</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>HSP19-II</td>
<td>√</td>
<td>√</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>Dehydrin</td>
<td>–</td>
<td>√</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>USP</td>
<td>–</td>
<td>√</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>S78</td>
<td>–</td>
<td>√</td>
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<td>na</td>
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<tr>
<td>S80</td>
<td>–</td>
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<td>Genes regulated by conditioning followed by chilling</td>
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<tr>
<td>FAD2</td>
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<td>LTP</td>
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<td>√</td>
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<td>ADH</td>
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<td>S28</td>
<td>–</td>
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<td>S34</td>
<td>√</td>
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</tr>
<tr>
<td>S41</td>
<td>–</td>
<td>√</td>
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</table>

* na, not affected by the HWB or conditioning treatments.

**Fig. 6.** A model describing the HWB-responsive and conditioning-responsive pathways involved in the acquisition of chilling tolerance in grapefruit. The HWB and conditioning treatments are capable of activating the expression of specific genes by themselves, without further exposure to chilling, but the majority of activated genes require further exposure to chilling. The HWB treatment followed by chilling activated the expression of various stress genes, whereas the conditioning treatment followed by chilling activated the expression of lipid modification enzymes. In addition, either treatment activates the expression of ADH, a translation initiation factor similar to SUI1, a chaperonin, and another clone (S34) with an unknown function.
1990; Van Breusegem et al., 1999), and glucanases are involved in cell wall softening and pathogen defence responses (Kauffmann et al., 1987; Brummel et al., 2004). In many horticultural commodities including citrus, ethylene enhances the development of CI during low temperature storage (Lafuente et al., 2001; Porat, 2004). Interestingly, in the present study, it was found that HWB down-regulated the expression of the EIN2 gene, a transducer of the ethylene response (Alonso et al., 1999), and, thus, may have inhibited the development of ethylene-promoted CI.

The identified conditioning-induced chilling tolerance-responsive pathway comprises the expression of FAD2 and LTP, which encode enzymes involved in lipid metabolism. The FAD2 gene encodes a microsomal 18:1 desaturase that is required for survival of Arabidopsis plants under chilling temperatures (Miquel et al., 1993). The possible involvement of the FAD2 gene in conditioning-induced chilling tolerance responses is further supported by other reports that conditioning treatments increased fatty acid desaturation and chilling tolerance in tobacco and grapefruit (Nordby et al., 1987; Kodama et al., 1999). LTPs are involved in membrane biogenesis and the transport of phospholipids (Somerville and Browse, 1991; Kader, 1996). In addition, LTPs are induced in plant cells upon exposure to biotic and abiotic stresses; in some cases they were reported to exhibit antifreeze activity and, thus, were hypothesized to be involved in conferring frost tolerance (Hon et al., 1995; Wu et al., 2004). Hereby, it is suggested that LTPs may also participate in conditioning-induced chilling tolerance responses in grapefruit.

The group of genes induced by chilling that follows either the HWB or the conditioning treatment, but not by chilling alone most probably encode major chilling tolerance proteins required to protect the fruit upon exposure to low temperatures. These genes include S28, which encodes a translation initiation factor similar to SU11. It was reported that besides initiating transcription, the SU11 protein may also be involved in repairing impaired mRNAs (Cui et al., 1999). In sum, it is possible that the SU11 protein may be required to govern translation under low temperature conditions. The HWB and conditioning treatments also increased the expression of a chaperonin gene; these, like HSPs, are required to maintain protein structure and function under stress conditions (Yamada et al., 2002; Wang et al., 2004). Finally, it was found that both treatments increased the expression of a specific glutathione-dependent formaldehyde dehydrogenase (ADH, class III), known to be involved in detoxification of aldehydes (Martinez et al., 1996). Further support for the hypothesis that ADH activity may be required for chilling tolerance comes from a recent study of maize, which demonstrated that plants containing double-null mutations in adh1 and adh2 developed more CI than wild-type plants, following exposure to 2 °C (Peters and Frankel, 2004).

Overall, a general model illustrating the various HWB-responsive and conditioning-responsive chilling tolerance pathways in grapefruit peel tissue is presented in Fig. 6. It can be seen that HWB and conditioning treatments activated several stress defensive genes by themselves (HSP19-I, II, FAD2, S8, and S34), but the majority of the HWB- and conditioning-regulated genes were induced only following subsequent exposure to chilling. Furthermore, the HWB chilling tolerance-responsive pathway activated mainly expression of stress-related genes, whereas the conditioning chilling tolerance-responsive pathway activated expression of genes involved in fatty acid lipid metabolism (Fig. 6). It should be noted that in most cases the combined application of both HWB and conditioning treatments had similar effects on gene expression levels as compared with each treatment alone (Fig. 5A–C). However, the transcript levels of two clones, S49 and S70, were specifically induced by the combined application of both treatments but not by each one of them alone (Fig. 5D). These observations may explain in part why the combined application of HWB and conditioning together was more effective in reducing CI as compared with each treatment alone (Fig. 2).

Finally, an important observation from the gene expression studies is that the pre-storage hot water and conditioning treatments had only minor effects on gene expression patterns by themselves, but rather had ‘potentiation’ or ‘priming’ effects, and enabled the fruit to activate most of its defence responses after subsequent exposure to chilling (Table 3). Similar priming effects have been reported previously regarding the mode of action of salicylic acid in promoting the induction of Hsp70/Hsc70 in response to heat in tomato, and in enhancing ion transport following elicitation in parsley cells (Crunje and Bornman, 1999; Katz et al., 2002). Furthermore, it was recently demonstrated that β-aminobutyric acid enhanced pathogen defence and abiotic stress tolerance in Arabidopsis and grapevine by priming the plants’ responses towards exposure to stresses (Hamiduzzaman et al., 2005; Ton et al., 2005). Finally, it has been shown that exposure to short days potentiated low temperature-induced gene expression during cold acclimation in silver birch (Puhakainen et al., 2004). Thus, potentiation or priming effects may provide an important mode of action in conferring plant stress tolerance by various treatments.

Altogether, in this study, evidence for the existence of two different chilling tolerance-responsive pathways in grapefruit has been uncovered, and it has been shown that pre-storage HWB and conditioning treatments potentiate the fruit peel tissue enabling it to respond adequately to chilling by enhanced expression of various low-temperature defensive genes (Table 3).
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References


Heat and conditioning synergistically prevent chilling injuries


